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Phylogenetic Implications of Phasmid Absence in Males of Three Genera in Heteroderinae[1]

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Abstract: Absence of the phasmid was demonstrated with the transmission electron microscope in immature third-stage (M3) and fourth-stage (M4) males and mature fifth-stage males (M5) of *Heterodera schachtii*, M3 and M4 of *Verutus volvingentis*, and M5 of *Cactodera eremica*. This absence was supported by the lack of phasmid staining with Coomassie blue and cobalt sulfide. All phasmid structures, except the canal and ampulla, were absent in the postpenetration second-stage juvenile (J2) of *H. schachtii*. The prepenetration *V. volvingentis* J2 differs from *H. schachtii* by having only a canal remnant and no ampulla. This and parsimonious evidence suggest that these two types of phasmids probably evolved in parallel, although ampulla and receptor cavity shape are similar. Absence of the male phasmid throughout development might be associated with an amphimictic mode of reproduction. Phasmid function is discussed, and female pheromone reception ruled out. Variations in ampulla shape are evaluated as phylogenetic character states within the Heteroderinae and putative phylogenetic outgroup Hoplolaimidae.

Key words: amphimixis, ampulla, cell death, *Cactodera eremica*, *Heterodera schachtii*, Heteroderinae, parallel evolution, parthenogenesis, phasmid, phylogeny, ultrastructure, *Verutus volvingentis*.

Phasmid sensory organs on the tails of secernentean nematodes are sometimes notoriously difficult to locate with the light microscope (18). Because the assignment of uncertain genera to classes Secernentea or Adenophorea depends in part on this taxonomically important character, phasmid obscurity can make this decision difficult. In addition, the phasmid may be secondarily lost altogether in some species which, on the basis of other characters, clearly belong to Secernentea (22,33). Where phasmids are clearly present in juveniles and often in females of a species, phasmids have sometimes been reported to be absent in males of the same species (9). This was supported by use of scanning electron microscopy (SEM) on the cockroach parasite, *Hammerschmidtiella diesingi* Chitwood, 1932, where phasmid openings are absent in males (44). The SEM did not show phasmid openings in the males of most genera within the plant-parasitic Heteroderinae, except *Meloidodera* (24) and perhaps *Cryphodera* (10) and *Zelandodera* (43).

Recent transmission electron microscope (TEM) observations of *Meloidodera* males showed phasmids to be present in all stages, and their variability was described (7). Because sufficient reliable characters are lacking for phylogenetic evaluation in Heteroderinae (5), further research is needed to determine the variability of the phasmid within the family and specifically confirm the absence of male phasmids with TEM.

Phasmids were not observed on males of *Heterodera schachtii* Schmidt, 1871 with SEM (24), but phasmids have been described in the literature (16). Males of *Verutus volvingentis* Esser, 1981 and *Cactodera eremica* Baldwin & Bell, 1985 do not have phasmids visible with SEM (24). *Verutus* represents a genus of uncertain position in the subfamily because the mixture of some ancestral and other highly derived characters confounds parsimonious accommodation to phylogenetic schemes (5).

There may be intermediate phasmid character states throughout development forming a transition series within the Heteroderinae. Any evidence for functional associations with stages in development should be considered in evaluating reli-

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ability and homology of the potential characters, including phasmid loss. There is a need for study, not only of the presence or absence of the phasmid, but of the morphology of the characteristic phasmid structures such as the ampulla, neuron, socket and sheath cell.

Materials and Methods

Verutus volvingentis second-stage juveniles (J2), fourth-stage males (M4), and mature adult males (M5) were extracted with a warm mist from buttonweed (*Diodea virginiana*) roots. The culture was maintained in the University of California, Riverside greenhouse from specimens supplied from Orlando, Florida, by D. T. Kaplan. The posthatch stage of *Verutus* is considered a J2 for comparison with other heteroderines, although Esser (14) was unable to detect a molt within the egg. The different stages were identified by superimposed ecdysed cuticles as well as by the tail and general body morphology (13,14).

Heterodera schachtii sugar beet populations from Santa Maria, California, and Great Barton, England, were maintained on sugar beet in the greenhouse. The English population, established from cysts provided by A. R. Stone, was believed to be from similar populations used by Franklin (16) in illustrating a male phasmid. Nematodes were inoculated to sterile sugar beet (*Beta vulgaris*) or rape seed (*Brassica napus*) plants in Gamborg's medium (pH 6.5) and Gelrite supplemented with vitamins (28). Plants were grown under standard Gro-lux lamps at 400 lumens for approximately 1 week before being inoculated with nematodes. Nematodes were hand picked into Beem capsule baskets containing previously boiled tap water, placed in 0.0004% $HgCl_2$ (23) for 3–20 hours, and transferred to one-half strength saturated rifampicin (6) for 10 minutes. Nematodes were then directly pipetted onto the culture medium.

Heterodera schachtii stages were dissected from the roots with a scalpel and needle, and the stages were identified by body morphology and surrounding cuticles (27). Nematodes were fixed, infiltrated, sectioned, and stained for TEM by previously reported methods (7). The following specimens were sectioned: *H. schachtii*—eight M5, three M4, two M3, and one postpenetration J2 (English population); *V. volvingentis*—four M5, four M4, and three J2; *C. eremica*—two M5.

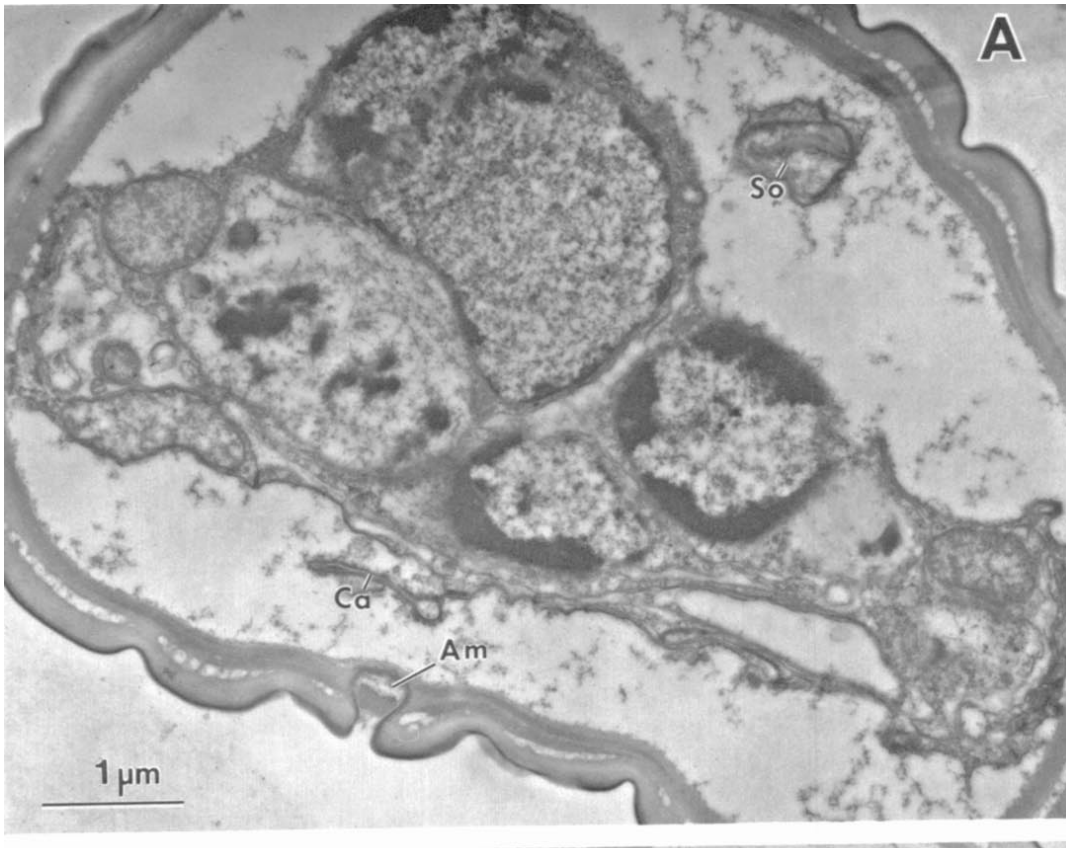
Two procedures were used to stain adult male phasmids of *V. volvingentis* and *H. schachtii* for light microscopy (LM). One was an adaptation of a cobalt sulfide–alkaline phosphatase method for insect sensory organs using an acetone dip followed by cobalt chloride and ammonium sulfide (30). The second method used Coomassie blue in acetic acid–methanol, where the specimens were subjected to butyl acetate in a ring of Zut (26). Both procedures were lethal to the nematodes.

Results

No phasmid or any part of its structure could be seen with TEM in M3, M4, and M5 specimens of either population of *H. schachtii*, in M4 and M5 of *V. volvingentis*, or in M5 of *C. eremica*. Both phasmid stains for LM gave negative results for the J2 and M5 stages of *H. schachtii* and *V. volvingentis*. Phasmids in the J2 and M5 of *Meloidodera floridensis* and *Meloidogyne incognita*, which were used as controls, were distinctly stained.

Heterodera schachtii: The postpenetration J2 has a flask-shaped ampullar opening (Figs. 1B, 3G); a slightly curved, ladle-shaped canal (Fig. 1A); and remnants of the socket cells, canals, and sheath cells (Fig. 1A, B). There is no indication of a neuron or cell nuclei. The cellular retraction from the cuticle indicates that molting has begun (Fig. 2A, B).

Verutus volvingentis young J2 has a bottle-shaped ampulla opening (Figs. 2B, 3F) with a plug of dense secretion traversing the outer pore to the outside of the body wall cuticle (Fig. 2B). A canal is visible within a single socket cell (Fig. 2A, C). The distal region of the sheath cell surrounds a narrow, ladle-shaped end apparatus (canal extension), which merges into the beginning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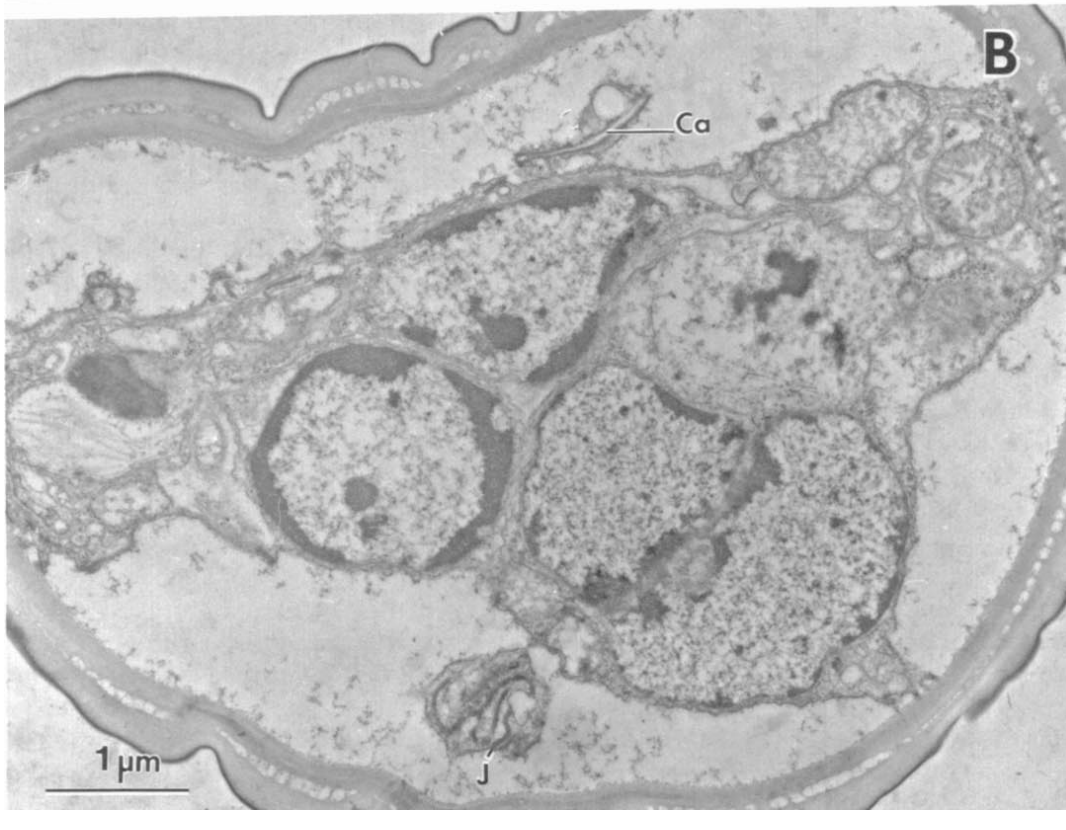
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of a small circular receptor cavity. Peripheral to the center circle of the receptor cavity, approximately eight long, curved lamellae surround the dendrite (Fig. 2A). The lamellae have dense deposits near the central cavity, and the sheath cell contains moderately dense glycogen deposits. The socket cell has less glycogen than the sheath cell and a few mitochondria.

Older J2 specimens (Fig. 2D) exhibit extensive cellular degeneration similar in appearance to the degenerating tails in older *H. schachtii* J2. Only an inflated canal and possibly some of the condensed lamellar membranes from the degenerate sheath cell are visible. Neither an ampulla nor dendrite is apparent.

Discussion

The sheath cell of *Verutus* has a receptor cavity and lamellar form very much like those of *Heterodera* (2). The ampulla of *Verutus* shows a greater similarity to *Heterodera* than *Meloidodera* (7), although it is not identical. A slightly different pattern of phasmid degeneration is seen between *Verutus* and *Heterodera*, in that the ampulla is not seen in the degenerating phasmid of *Verutus* but occurs throughout the J2 of *Heterodera*.

Although *Verutus* still appears to have a pore-like phasmid similar to that of *Heterodera*, there is a difference in the sequence of structures lost. Therefore parallel evolution may have produced two types of pore-like phasmids. In the future these may be designated as two distinct character states, such as Pore A and Pore B. The two character states occur within the ampulla shape character.

Despite its presence in *Meloidodera* spp. (7), the absence of a phasmid in *Verutus* and *Heterodera* males provides further evidence that *Verutus* and *M. charis* are not a monophyletic group, which is in direct contrast to the proposal by Ferris (15). This is further supported by characters unique to *Verutus*, including highly derived lip patterns, diminutive host syncytium, and multiple-B-layer of the body wall (5). Consideration of 19 reliable characters in a computerized phylogenetic analysis placed *Verutus* as an outgroup of all other Heteroderinae (5). It has been proposed that *Verutus* is part of the Rotylenchulidae (Hoplolaimidae) (35), a hypothesis that will require further testing (4).

Loss of the phasmid in *H. schachtii* after hatching clarifies the developmental polarity of the two forms of the J2 phasmid originally reported in this species. Type A is a relatively rare, well-developed phasmid; type B is a poorly developed phasmid (2). We have shown that the phasmid changes with time of development and that the type B phasmid represents an intermediate stage between the well-developed type A phasmid of young J2 and the degenerate remnant phasmid of the older J2. Instead of the cellular features typical of a normal programmed cell death (21,29), the degenerate type B phasmid contains large vacuoles (2). With further study, this vacuolate degeneration may prove to be similar to the type of cell death seen in *Caenorhabditis elegans* Maupas, 1899 mec-4 and deg-1 touch cell mutants (8).

Loss of phasmids in Heteroderinae may be under hormonal control, as is the case for certain organs in insects and other organisms (1,40). The juvenile hormone-ecdysone balance responsible for morphogenetic variation in insects may be influenced by ovarial ecdysone or by sex hormones (36), sometimes affecting programmed neuronal cell death (39).

Although phasmids were indicated in the taxonomic description of *H. schachtii* males (16), the phasmid was not described or illustrated on males in a description of the

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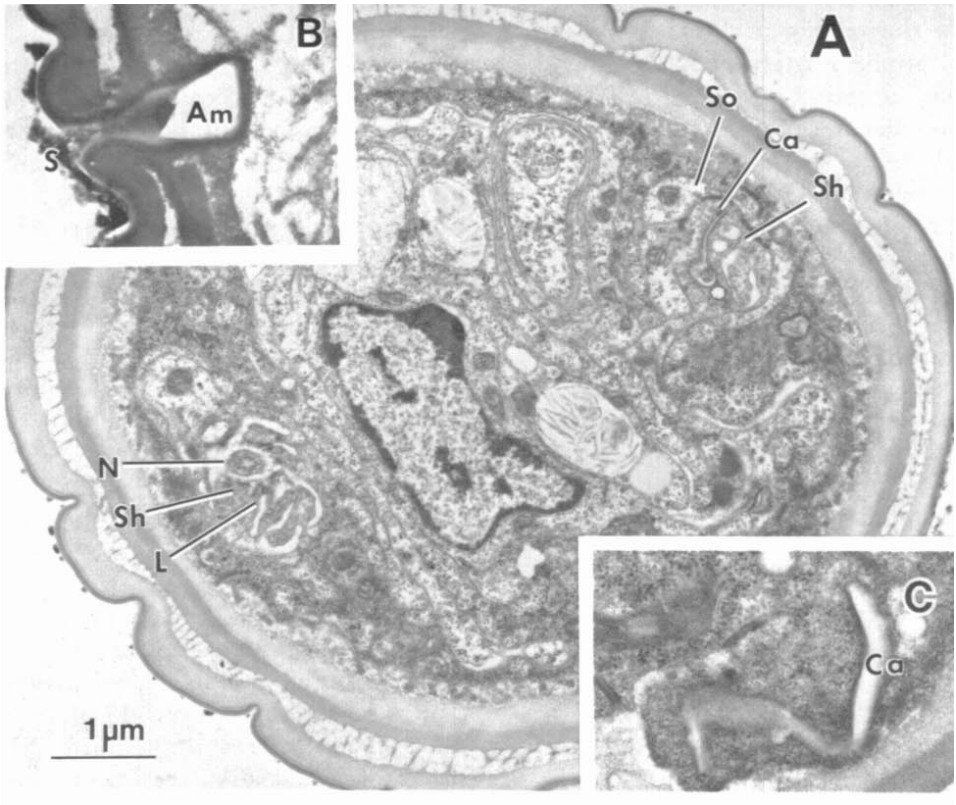
Fig. 1. *Heterodera schachtii* postpenetration J2 from sugar beet root. A) Cross section at level of ampulla (Am). B) Cross section with canal (Ca) and sheath cell–socket cell intercellular junction interface (J). So = socket cell.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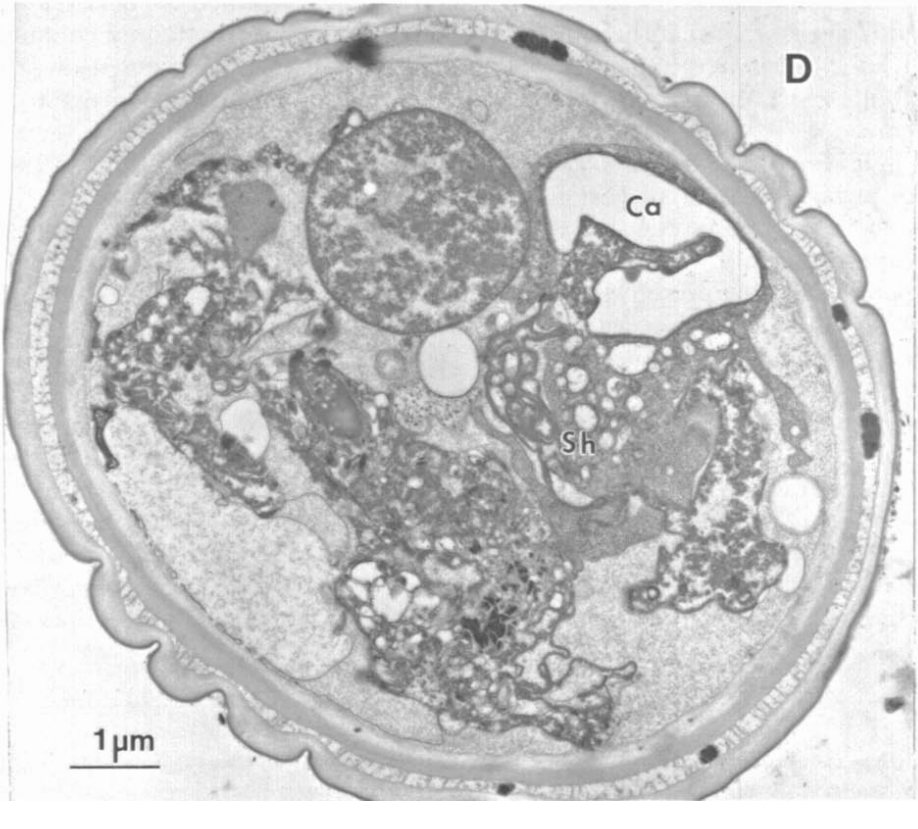
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life history (27). This absence, which our studies have confirmed, is noteworthy in relation to the proposal that the male phasmid may detect female sex pheromone in this species (41), a suggestion that is clearly not tenable.

The osmotic detection hypothesis of phasmid function (7) can better accommodate phasmid loss in later stages. Osmotic regulation is a primary activity within the egg before hatch (25), and perhaps the phasmid may be essentially a juvenile organ for osmotic detection within the egg. Where the phasmid is retained through development, it may have less adaptive value than in young nematodes.

In certain life stages of some species, the phasmid may not be retained when hormonal events associated with amphimictic sex determination begin. This hypothesis is based upon nematode descriptions in the literature of species that lack adult male phasmids and that are proven or assumed to require males for reproduction (amphimixis). This reproductive mode is true of *H. schachtii* (18,38). It is probably also true of *Verutus* which has a high male sex ratio and reportedly a close phylogenetic relationship with the amphimictic *Rotylenchulus* (35). *Scutellonema cavenessi* Sher, 1964 is amphimictic (12) and no phasmids are described in the male (32). Species of *Hammerschmidtiella* (44) and filarial worms (37) also have males that lack phasmids, and the high proportion of males in some species suggests association with reproduction by amphimixis. In the citrus nematode, *Tylenchulus semipenetrans* Cobb, 1913, no phasmid is visible with SEM in the early differentiated J2 males and females (unpubl.). In contrast, in nematodes where the adult male phasmid is present, such as *Meloidodera, Meloidogyne,* and *Caenorhabditis,* autotokous (male-independent) reproduction occurs by parthenogenesis or hermaphroditism (38,39). Even within the mainly amphimictic Heteroderinae, a detailed description of a phasmid is given for the parthenogenetic male of *Cactodera betulae* Hirschmann & Riggs, 1969 (20).

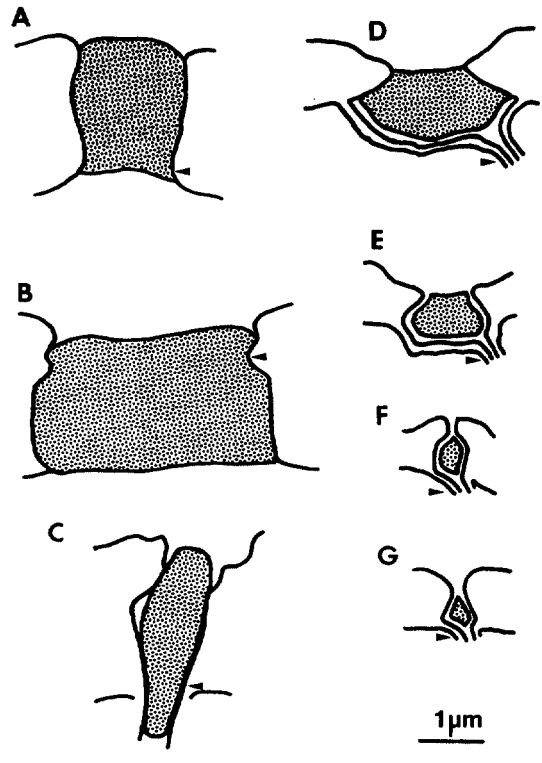

FIG. 3. Phasmid ampullae in some Hoplolaimidae (A–C) and Heteroderinae (D–G). Construction designated by arrows. A) *Scutellonema brachyurum* (after Wang and Chen [41]). B) *Hoplolaimus* sp. (after Coomans and De Grisse [10]). C) *Rotylenchus robustus* (after Coomans and De Grisse [10]). D) *Meloidodera floridensis* (after Carta and Baldwin [7]). E) *Meloidodera charis* (after Carta and Baldwin [7]). F) *Verutus volvingentis.* G) *Heterodera schachtii.*

Absence of the male phasmid is generally associated with, and may be causally related to, sexual differentiation in certain amphimictic nematodes, whereas the presence of a phasmid in males is generally associated with autotoky (either parthenogenesis or hermaphroditism). Autotoky is

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FIG. 2. *Verutus volvingentis* J2. A) Cross section of young J2 at level of canal (Ca). B) Enlargement of ampulla (Am). C) Enlargement of canal. D) Cross section of old J2 showing remnant of canal and sheath cell (Sh). L = lamellae, N = neuron, S = dense secretion, So = socket cell.

considered a derived condition and phylogenetically a dead end (38). Outgroup comparison might support the argument that absence of phasmids, and correlated amphimixis, in adult heteroderine males is conserved from amphimictic Secernentea and that phasmid presence in males is a derived condition. Therefore within Secernentea autotoky and associated male phasmid presence might represent a derived rather than an ancestral condition.

There is a large amount of adenosine triphosphate normally present in sensory lamellar ensheathing cells (17), and there would be a selective advantage to amphimictic males conserving this energy for multiple matings. This would be particularly true for males of Heteroderinae which are generally believed to not feed. If the phasmid is an auxiliary osmotic detector in heteroderines, it may be less important to a male which might not smother its osmotically sensitive anterior amphids by feeding. The phasmid would then be of adaptive value to internally feeding parasitic stages.

The proposed derived condition of phasmids in males has important implications on the phylogenetic position of *Meloidodera*. The presence of a male phasmid as seen in *Meloidodera* and described for *Cryphodera* (10) could represent a shared, derived character which would thus support monophyly of these genera, excluding *Verutus*. If proven, this monophyly would justify designation of a higher taxon such as Meloidoderini for these two genera (5).

The ampullae of *Meloidodera* and other Heteroderinae vary in size and shape through development and between species (7). This is also true in the outgroup, Hoplolaimidae, where the ampulla may be large and columnar or reduced in width and slightly constricted in shape (Fig. 3A–C). In *Hoplolaimus* a slight constriction occurs on both sides of the outermost region of the opening. The scutellum opening of *Scutellonema* is about half that of *Hoplolaimus*, but the constriction is closer to the inner base of the plug (41). The narrower and deeper opening of *Rotylenchus* (Fig. 3C) maintains this constriction at the base of the plug (11).

The phasmids of Heteroderinae (Fig. 3D–G) possess a smaller opening relative to ampulla width with a more pronounced flare and constriction closer to the base of the ampulla than the Hoplolaimidae (Fig. 3A–C). The bottle-shaped ampulla of *Verutus* (Fig. 3F) is an intermediate form between that of the Hoplolaimidae and the Heteroderinae. It represents a relatively ancestral phasmid shape within the Heteroderinae. Aside from these distinctive characteristics of Hoplolaimidae and Heteroderinae, two general shapes of ampullae can be distinguished within both taxa. Ratio of the opening is either wide relative to its depth (0.9–2.5) as in *Scutellonema, Hoplolaimus,* and *Meloidodera,* or it is narrow (0.5) relative to its depth as in *Rotylenchus, Verutus,* and *Heterodera* (Fig. 3).

The opening in *Rotylenchus* is narrow relative to its depth and in this respect it is similar to the smaller bottle-shaped ampulla of *Verutus* and the flask-shaped ampulla of *Heterodera*. However, *Rotylenchus* is believed to be phylogenetically divergent relative to *Rotylenchulus* and Heteroderinae which may have a more recent common ancestor (38). The similarity in the shape of *Rotylenchus* with *Heterodera* and *Verutus* is likely to have arisen by parallel evolution, whereas we might predict that *Rotylenchulus* would have a shape intermediate between that of *Hoplolaimus* and *Meloidodera*. Thus, the pore phasmid would exist in parallel lines in the ancestral Hoplolaimidae and the derived Heteroderinae.

These ampullar forms indicate that the distinctions between the LM descriptions of lens-like, scutellum-like, and pore-like phasmids are actually more gradual transitions as shown by TEM. With LM a lens can be distinguished from a scutellum, but a small lens cannot be distinguished from a pore.

Critical studies of the pore-like phasmid of *Hylonema* and a new genus *Ekphymatodera* (4) may test the hypotheses of parallel evolution with the pores of derived cyst-forming genera. On the basis of parsi-

mony, one could predict that the phasmid opening of *Ekphymatodera* and *Hylonema* is a modified lens as in *Meloidodera charis* (3), and that these small phasmid openings are not homologous with the pore phasmid of cyst-forming genera. This would further support the notion that phasmid terms "lens" and "pore" reflect the limitations of light microscopy.

The phasmid is a complex structure of diagnostic and evolutionary characters, including ampulla size and shape, presence or absence in males, and sheath cell membrane pattern. With our current knowledge of these structures, phasmids in *Verutus* show greater similarity to phasmids in *Heterodera* than to those in *Meloidodera*. Their relative value will become more apparent as we better understand the variability and phylogenetic polarity of these structures in the remainder of the Heteroderinae and its outgroups.

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